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Haloarcula quadrata sp. nov., a square, motile archaeon isolated from a brine pool in Sinai (Egypt)

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The motile, predominantly square-shaped, red archaeon strain 801030/1^T, isolated from a brine pool in the Sinai peninsula (Egypt), was characterized taxonomically. On the basis of its polar lipid composition, the nucleotide sequences of its two 16S rRNA genes, the DNA G+C content (60·1 mol%) and its growth characteristics, the isolate could be assigned to the genus *Haloarcula*. However, phylogenetic analysis of the two 16S rRNA genes detected in this organism and low DNA–DNA hybridization values with related *Haloarcula* species showed that strain 801030/1^T is sufficiently different from the recognized *Haloarcula* species to warrant its designation as a new species. A new species, *Haloarcula quadrata*, is therefore proposed, with strain 801030/1^T (= DSM 11927^T) as the type strain.

Keywords: *Haloarcula quadrata*, square bacteria, archaea, halophile

INTRODUCTION

Square bacteria were first observed in 1980 by Walsby in the Gavish Sabkha, a coastal brine pool in the Sinai peninsula, Egypt (Parkes & Walsby, 1981; Walsby, 1980). Walsby recognized these extremely thin, square-shaped structures as prokaryotes due to the presence of refractile gas vacuoles. Square bacteria have since been reported to occur in many thalassohaline hypersaline environments in which the salt concentration exceeds 200–250 g l^{-1}, and up to NaCl saturation. These include saltern crystallizer ponds in Israel (Oren, 1994; Oren *et al*., 1996), Spain (Guixa-Boixareu *et al*., 1996; Torrella, 1986), San Francisco Bay (USA) and Baja California (Mexico) (Javor *et al*., 1982; Stoeckenius, 1981).

The gas-vacuole-containing type of square bacteria observed by Walsby has never been obtained in culture. A single report of the successful isolation of this type of bacterium from a Spanish saltern was published by Torrella (1986). Unfortunately, this isolate has not been deposited in a culture collection and the culture was lost many years ago. Other attempts to isolate this intriguing type of prokaryote from a variety of hypersaline environments (the Gavish Sabkha, salterns of Guerrero Negro, Baja California and San Diego, California) led to the isolation of 'box-shaped' pleomorphic red halophilic archaea, lacking gas vacuoles, which were classified in the genus *Haloarcula* on the basis of their polar lipid composition and 16S rRNA nucleotide sequences. These strains (species *incertae sedis* '*Haloarcula sinaiiensis*' and '*Haloarcula californiae*') (Javor *et al*., 1982) still await a more detailed characterization in order to define their taxonomic position. The triangular and rhomboid cells of *Haloarcula japonica* (Takashina *et al*., 1990) show some morphological similarity to these isolates.

Early isolation experiments yielded a motile, square bacterium from the Gavish Sabkha. This strain, which lacks gas vacuoles, was designated 801030/1^T. Its morphological characteristics and its interesting mode of motility were reported by Alam *et al*. (1984). Cells may possess a single flagellum or several flagella anchored from a single or from several locations on the

Abbreviations: PG, PGP, PGS, the diether derivatives of phosphatidylglycerol, the methyl ester of phosphatidylglycerophosphate and phosphatidylglycerosulfate, respectively; TGD-2, 1-*O*-[β-D-glucose-(1′ → 6′)-α-D-mannose-(1′ → 2′)-α-D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol.

The DDBJ accession numbers for the 16S rRNA sequences of strain 801030/1^T are AB010964 and AB010965.

cell surface. Several filaments may form a stable bundle that does not fall apart when changing from clockwise to anti-clockwise movement and vice versa. Instead of the three flagellar proteins present in flagella of *Halobacterium*, SDS-PAGE of isolated flagella of strain 801030/1^T showed the presence of a single flagellin band with an apparent molecular mass of 73 kDa.

We have performed a taxonomic characterization of the square motile strain 801030/1^T. On the basis of its growth characteristics, its polar lipid composition and the nucleotide sequences of its 16S rRNA genes, the isolate could be assigned to the genus *Haloarcula*. However, based on phylogenetic analysis of the two different 16S rRNA genes detected in this organism and the low DNA–DNA hybridization values with the other *Haloarcula* species, the isolate is sufficiently different from the recognized *Haloarcula* species to warrant its designation as a new species.

METHODS

Bacterial strains. A culture of isolate 801030/1^T (Alam *et al.*, 1984) was kindly supplied by M. Kessel (Bethesda, MD, USA). For comparison, reference strains of halophilic archaea were included in this study, as listed in Table 1.

Cultivation conditions. The standard growth medium used for strain 801030/1^T and other *Haloarcula* strains (Oren *et al.*, 1988) contained (in g l^{-1}): NaCl, 206; $MgSO_4 . 7H_2O$, 36; KCl, 0·373; $CaCl_2 . 2H_2O$, 0·5; $MnCl_2$, 0·013 mg l^{-1}; and yeast extract, 5 (pH 7). Other halophilic archaea used as reference strains were grown in this medium or other suitable media, as required. The growth medium was modified with respect to salt concentration, yeast extract content, addition of other nutrients, buffers, antibiotics, etc., as specified in the experiments. For growth experiments at different pH values, the buffers MES (in the pH range 5·0–5·5), PIPES (pH 6–7) and HEPES (pH 7·5–9·0) were added at a final concentration of 25 mM. These strains were routinely grown with shaking at 35 °C in 100 or 250 ml Erlenmeyer flasks containing 30 or 100 ml medium, respectively. Growth was assessed by measuring the OD_{600} of the cultures.

Phenotypic characterization. Unless specified otherwise, tests for phenotypic properties were carried out as indicated in the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Degradation of Tween and gelatin was tested as recommended by Gutiérrez & González (1972). Appropriate positive and negative controls were included in all tests performed.

Formation of acids from different sugars was tested in media in which the yeast extract concentration was reduced to 1·0 or 0·5 g l^{-1}, amended with 0·5 g NH_4Cl l^{-1} and 5 g of the sugar tested l^{-1} (autoclaved separately). To examine growth stimulation by sugars and other compounds, this medium was buffered at pH 7·0 by including 20 mM PIPES. To test for the ability of single carbon sources to support growth, yeast extract was omitted, and the compound to be tested was added at a concentration of 5 g l^{-1}, together with 1 g NH_4Cl l^{-1} and 0·05 g KH_2PO_4 l^{-1} and 20 mM PIPES. The outcome was considered positive when growth was obtained in at least four successive transfers in this medium.

Growth and gas formation with nitrate as electron acceptor were tested in 100 ml glass-stoppered bottles completely filled with growth medium to which 5 g $NaNO_3$ l^{-1} was

Table 1. DNA relatedness among strain 801030/1^T and other halophilic archaea studied

DNA–DNA relatedness values are mean results of at least three independent determinations, which generally did not differ by more that 5%. ^{T}, Type strain.

Organism	Percentage hybridization with ^{3}H-labelled DNA from strain 801030/1^T
Haloarcula strain 801030/1^T	100
Haloarcula vallismortis ATCC 29715^T	20
Haloarcula marismortui ATCC 43049^T	<10
Haloarcula hispanica ATCC 33960^T	22
Haloarcula japonica JCM 7785^T	34
Haloarcula argentinensis ATCC 29841^T	14
Haloarcula mukohataei DSM 11483^T	<10
'*Haloarcula sinaiiensis*' ATCC 33800	<10
Haloferax gibbonsii ATCC 33959^T	18
Haloferax volcanii NCIMB 2012^T	<10
Haloferax mediterranei ATCC 33500^T	<10
Halobacterium salinarum DSM 3754^T	18
Halorubrum saccharovorum ATCC 29252^T	15
Halorubrum lacusprofundi DSM 5036^T	<10
Halorubrum sodomense ATCC 33755^T	<10
Halorubrum distributum JCM 9100^T	<10
Halobaculum gomorrense DSM 9297^T	<10
Natrialba asiatica JCM 9576^T	<10

added and which included an inverted test tube to observe the formation of gas. Anaerobic growth in the presence of 5 g L-arginine.HCl l^{-1} was tested in completely filled 25 ml stoppered tubes. Controls without nitrate or arginine were included and all incubations were performed in the dark. *Haloarcula marismortui* was used as a positive control for the formation of nitrite and gas from nitrate, and *Halobacterium salinarum* served as a positive control for anaerobic growth on arginine.

Polar lipid characterization. Cell pellets from 20 ml culture were suspended in 1 ml 4 M NaCl and extracted with 3·75 ml methanol/chloroform (2:1, v/v) for 4 h. The extract was centrifuged and the pellet was re-extracted with 4·75 ml methanol/chloroform/water (2:1:0·8, v/v). Chloroform and water (2·5 ml each) were added to the combined supernatants to achieve phase separation and, after centrifugation, the chloroform phase was collected and dried in a vacuum desiccator. Lipids were redissolved in a small volume of chloroform, applied to silica gel plates (20 × 20 cm; Sigma) and separated by single development with chloroform/methanol/acetic acid/water (85:22·5:10:4, v/v). Lipid spots were detected by spraying with the following reagents: 0·5% α-naphthol in 50% methanol, followed by 5% H_2SO_4 in ethanol, and heating of the plates at 150 °C (allowing detection of glycolipids); 0·2% $CeSO_4$ in 0·5 M H_2SO_4, followed by heating at 150 °C (a general lipid stain, allowing differentiation of glycolipids from other lipids by colour); and ammonium molybdate/sulfuric acid reagent (for the detection of phospholipids) (Oren *et al.*, 1996; Torreblanca *et al.*, 1986).

Extraction of genomic DNA and determination of DNA base composition. Cells were harvested, washed in 25% NaCl and suspended in 0·15 ml NaCl/0·1 M EDTA buffer, pH 8·0. Lysis was accomplished at 60 °C for 10 min after adding SDS at a final concentration of 2% (w/v). The DNA was extracted and purified by the method of Marmur (1961). The purity was assessed from the A_{260}/A_{280} and A_{230}/A_{260} ratios (Johnson, 1994).

The DNA G+C content was determined from the midpoint value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) in 0·1× SSC buffer (0·15 M NaCl buffered with 0·015 M trisodium citrate, pH 7·0) and was obtained with a Perkin-Elmer UV-Vis 551S spectrophotometer at 260 nm. The instrument was programmed for temperature increases of 1·0 °C min^{-1}. The T_m was determined by a graphic method described by Ferragut & Leclerc (1976) and the G+C content was calculated from this temperature by using the equation of Owen & Hill (1979). The T_m value of reference DNA from *Escherichia coli* NCTC 9001 was taken as 74·6 °C in 0·1× SSC (Owen & Pitcher, 1985).

Preparation of labelled DNA and DNA–DNA hybridization experiments. DNA was labelled by the multiprime system with a commercial kit (RPN 1601 Y; Amersham) with deoxy[1′2′5′-^{3}H]cytidine 5′-triphosphate (Amersham). The mean specific activity obtained with this procedure was $8·4 \times 10^6$ c.p.m. μg^{-1} DNA. The labelled DNA was denatured prior to hybridization by heating at 100 °C for 5 min and then placed on ice.

DNA–DNA hybridization studies were performed by the competition procedure of the membrane method described by Johnson (1994). Competitor DNAs were sonicated (Braun) at 50 W for two 15 s time intervals. Membrane filters (HAHY; Millipore) containing reference DNA (25 $\mu g\ cm^{-2}$) were placed in 5 ml screw-cap vials that contained the labelled, sheared, denatured DNA and the denatured, sheared competitor DNA. The ratio of the concentrations of competitor to labelled DNA was at least 150:1. The final volume and concentration were adjusted to 140 µl, 2× SSC and 30% formamide. Hybridization was performed for 18 h in a water bath (Grant Instruments) with slow shaking at 56 °C, which is within the limits of validity for the filter method (De Ley & Tijtgat, 1970), in triplicate. After hybridization, the filters were washed in 2× SSC at the optimal renaturation temperature (56 °C). The radioactivity bound to the filters was measured in a liquid scintillation counter (Beckman Instruments) and the percentage homology was calculated according to Johnson (1994). At least three independent determinations were carried out for each experiment.

Analysis of 16S rDNA. The nucleotide sequences of the 16S rRNA genes were determined as described by Kamekura & Seno (1993).

RESULTS

Cells of the halophilic archaeon strain 81030/1^T were predominantly square-shaped, somewhat pleomorphic, flat cells under all growth conditions tested when sufficient magnesium was present in the medium (see below). Cells measured between 2 and 3 µm in diameter (Fig. 1). Gas vacuoles were never observed. Cells were motile by means of one or more flagella as documented by the dark-field microscopy pictures and electron micrographs presented by Alam *et al.* (1984). Cells stained Gram-negative. Colonies on agar plates were small (less than 1 mm in diameter, even after prolonged incubation), entire, smooth and red-orange. Oxidase and catalase reactions were positive.

As for other halophilic members of the archaea, high salt concentrations were required for growth and for stabilization of the cells. Optimum growth was observed in the presence of 3·4–4·3 M NaCl in media containing 0·15 M Mg^{2+} and no growth was obtained below 2·7 M NaCl. At least 50–100 mM Mg^{2+} was required for optimal growth and for maintenance of the square morphology. Best growth was obtained at Mg^{2+} concentrations between 0·1 and 0·5 M in medium containing in addition 3·3 M Na^+. In the presence of

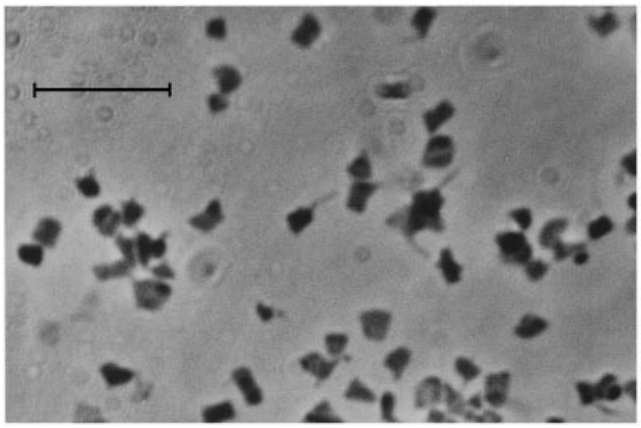

Fig. 1. Phase-contrast micrograph of a culture of strain 801030/1^T in standard growth medium. Bar, 10 µm.

1 M Mg^{2+}, growth was severely inhibited. When cells were suspended in NaCl concentrations lower than 0·5 M and in the absence of divalent cations, immediate lysis occurred and NaCl concentrations as high as 3·5 M were required to maintain the native cell shape. At intermediate NaCl concentrations (0·7–3·4 M), cells lost their native square or pleomorphic shape and turned into spheroplasts. A similar phenomenon has been reported previously in *Haloarcula marismortui* (Cohen *et al.*, 1983). At least 40 mM Mg^{2+} was required to prevent rounding of the cells and, in the presence of 100 mM $MgCl_2$, the native cell shape was maintained even when the cells were suspended in solutions lacking NaCl.

The optimum pH for growth was 6·5–7·0; no growth was observed below pH 5·9 and above pH 8·0. The optimum growth temperature was 50–53 °C; no growth was observed above 55 °C.

A number of sugars and other organic compounds stimulated growth in buffered media in which the yeast extract concentration was reduced to 0·05–0·10 %. Growth stimulation was observed by glucose, galactose, maltose, sucrose, mannitol, sorbitol, glycerol, succinate and pyruvate. Fructose, mannose, arabinose, ribose, xylose, lactose, methyl α-glucoside, acetate, DL-malate and citrate were not stimulatory. Glucose, sucrose and maltose were the only single carbon compounds which supported growth for more than five consecutive transfers in mineral medium. In unbuffered medium, acid was produced in the presence of glucose, galactose, sucrose, xylose and ribose. No or little acid formation was observed in the presence of fructose, mannose, arabinose, maltose, lactose, methyl α-glucoside, mannitol, sorbitol or glycerol.

Strain 801030/1^T grew anaerobically in the presence of nitrate with formation of nitrite and gas. No fermentative growth was demonstrated in media containing L-arginine.

Starch was hydrolysed. Tests for hydrolysis of gelatin, casein, Tween 20 and Tween 80 all yielded negative results. No indole was produced. The strain was sensitive to bacitracin, novobiocin and anisomycin (25 μg ml^{-1}) and resistant to penicillin, ampicillin, rifampicin, chloramphenicol, neomycin and erythromycin (all at 50 μg ml^{-1}).

The polar lipid pattern was characteristic for representatives of the genus *Haloarcula*, with diether derivatives of phosphatidylglycerol, the methyl ester of phosphatidylglycerophosphate and phosphatidylglycerosulfate (PG, PGP and PGS, respectively), and a single glycolipid (TGD-2, 1-*O*-[*β*-D-glucose-(1′ → 6′)-α-D-mannose-(1′ → 2′)-α-D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol) (Tindall, 1992).

The affiliation of the isolate with the genus *Haloarcula* was confirmed by 16S rRNA sequence comparisons. At least two 16S rRNA genes were detected, designated A (1470 bp) and B (1471 bp). The 801030/1^T A gene showed the highest similarity (98·2 %) with the *Haloarcula marismortui* A gene. The B gene showed a high degree of similarity with the *Haloarcula marismortui* B gene, the *Haloarcula vallismortis* A gene and the major gene of '*Haloarcula sinaiiensis*' (99·1, 99·0 and 99·1 % similarity, respectively). The sequences determined were aligned with those previously reported and used to reconstruct a phylogenetic tree. Positions with any deletions or of uncertain alignment were removed and the remaining 1440 positions were used to reconstruct the tree (Fig. 2).

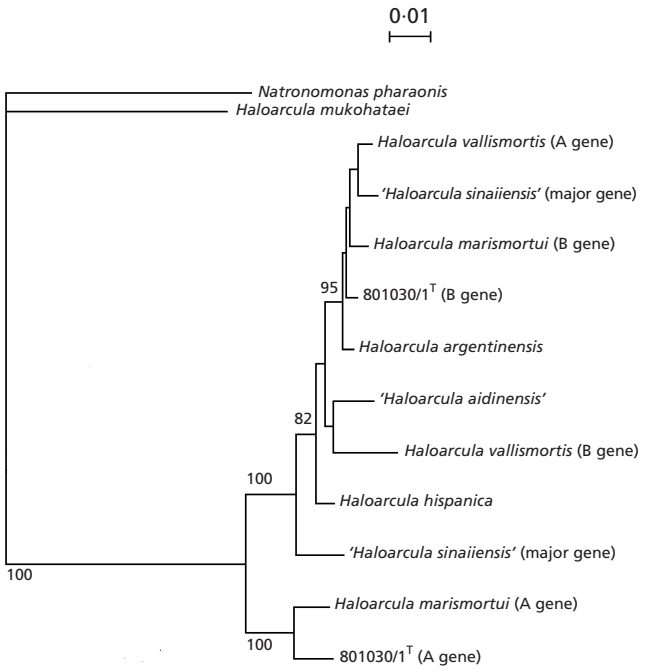

Fig. 2. Phylogenetic tree reconstruction of the genus *Haloarcula* by the neighbour-joining method (CLUSTAL W 1.5) with 16S rRNA gene sequences. The sequence data used were from the following strains (accession numbers are given in parentheses): *Haloarcula vallismortis* ATCC 29715^T A gene (U17593) and B gene (U68451); *Haloarcula marismortui* ATCC 43049^T A gene (X61688) and B gene (X61689); *Haloarcula argentinensis* CM 9737^T (D50849); *Haloarcula mukohataei* JCM 9738^T (D50850); '*Haloarcula sinaiiensis*' ATCC 33800 major and minor gene (D14129 and 14130, respectively); and '*Haloarcula aidinensis*' strain B02 (AB000563). Bootstrap values (1000 replicates) above 70 % are shown. Scale bar, 0·01 expected changes per site. *Natronomonas pharaonis* JCM 8858^T (D87971) was used as the outgroup.

DNA–DNA hybridization studies showed only low relatedness of DNA from strain 801030/1^T with other representatives of the genus *Haloarcula*, the highest value being 34 % with *Haloarcula japonica* JCM 7785 (Table 1).

The G+C content of strain 801030/1^T was 60·1 mol %, as determined by thermal denaturation (mean of three independent determinations).

DISCUSSION

The genus *Haloarcula* presently consists of the following six recognized species: *Haloarcula vallismortis* (González *et al.*, 1978), *Haloarcula marismortui* (Oren

Table 2. Some properties differentiating strain 801030/1^T from the validly described species within the genus *Haloarcula*

Strains: 1, strain 803010/3^T; 2, *Haloarcula vallismortis*; 3, *Haloarcula marismortui*; 4, *Haloarcula hispanica*; 5, *Haloarcula japonica*; 6, *Haloarcula argentinensis*; 7, *Haloarcula mukohataei*. +, Positive; −, negative; ±, variable; ND, no data available. Data were derived in part from Ihara *et al*. (1997), Oren *et al*. (1988), Takashina *et al*. (1990) and Tindall (1992).

Character	1	2	3	4	5	6	7
Dominant shape	Square	Rod	Pleomorphic	Short rod	Triangular	Triangular	Short rod
Motility	+	+	±	+	+	+	+
Hydrolysis of:							
Starch	+	+	+	+	−	ND	ND
Gelatin	−	−	−	+	−	ND	ND
Casein	−	−	ND	±	−	ND	ND
Tween 80	−	ND	ND	+	ND	ND	ND
TGD-2 glycolipid	+	+	+	+	+	+	−
DNA G+C (mol%)	60·1	64·7	61·8	62·7	63·3	62	65

et al., 1990), *Haloarcula hispanica* (Juez *et al*., 1986), *Haloarcula japonica* (Takashina *et al*., 1990), *Haloarcula argentinensis* and *Haloarcula mukohataei* (Ihara *et al*., 1997). In addition, there are three species *incertae sedis* which are still awaiting a valid description: '*Haloarcula sinaiiensis*', '*Haloarcula californiae*' (Javor *et al*., 1982) and '*Haloarcula aidinensis*' (Peijin *et al*., 1994; Xu *et al*., 1995). Strain 801030/1^T clearly belongs to this genus. This is apparent from its polar lipid composition, which is characterized by the triglycosyl glycolipid TGD-2, found thus far only in *Haloarcula* species as the sole glycolipid in all species described, with the exception of *Haloarcula mukohataei*. The latter species was placed in the genus *Haloarcula* on the basis of the similarity of its 16S rRNA gene sequence to the 16S rRNA sequences of other members of the genus. However, ten out of the sixteen signature bases of the genus *Haloarcula* are replaced by other bases in *Haloarcula mukohataei* (data not shown). It may therefore be justified to propose its transfer to a novel genus (Kamekura, 1999). Additional characteristics common to strain 801030/1^T and the hitherto described species of the genus *Haloarcula* are its relatively high temperature optimum for growth, its ability to grow on a number of simple sugars as sole carbon and energy source, and its flat somewhat pleomorphic morphology. Also, the property of motility is shared with most other *Haloarcula* species. Flagellar motility has been documented in *Haloarcula vallismortis* (González *et al*., 1978), *Haloarcula japonica* (Takashina *et al*., 1990), *Haloarcula hispanica* (Juez *et al*., 1986), *Haloarcula argentinensis* and *Haloarcula mukohataei* (Ihara *et al*., 1997). *Haloarcula marismortui* has been reported as being non-motile (Tindall, 1992), but Oren *et al*. (1988) described motile cells rotating around their axis. The G+C content of the DNA (60·1 mol%) is somewhat lower than that reported for the other *Haloarcula* species described (61·8–65·0 mol%). Table 2 presents a comparison of a number of phenotypic and genotypic properties of strain 801030/1^T and the validly described *Haloarcula* species.

Strain 801030/1^T contained more than one non-identical 16S rRNA gene. Also this property is common to representatives of the genus *Haloarcula*. Thus, *Haloarcula vallismortis*, *Haloarcula marismortui*, and '*Haloarcula sinaiiensis*' were reported to possess at least two heterogeneous 16S rRNA genes (Kamekura, 1999; Mylvaganan & Dennis, 1992). Both sequences confirm the affiliation of the strain with the genus *Haloarcula*, with a particularly high similarity with *Haloarcula marismortui*, *Haloarcula vallismortis*, and '*Haloarcula sinaiiensis*' (Fig. 2). Unfortunately, no complete 16S rRNA sequence of *Haloarcula japonica* has been published. Aligning the available partial sequence (D28872), encompassing 1424 bases including 88 where the base assignment was ambiguous, yielded similarity values of 96·3 and 94·4% with the 801030/1^T A and B genes, respectively.

DNA–DNA hybridization experiments (Table 1) showed only low degrees of relatedness of strain 801030/1^T with the recognized *Haloarcula* species, as well as with other halophilic archaea. Notably, the hybridization with DNA from *Haloarcula marismortui*, the organism showing the closest phylogenetic relationship based on 16S rRNA sequences, was extremely low. The highest DNA–DNA relatedness (34%) was measured with *Haloarcula japonica* DNA. However, this value is still far below the consensus value of 70% delineating the species level (Wayne *et al*., 1987). Such low DNA–DNA hybridization values do not provide any significant information on phylogenetic relationships.

The data presented above suggest that the square halophilic archaeon strain 801030/1^T belongs to the genus *Haloarcula*, but cannot be placed in any of the recognized species within that genus. Therefore, we propose a new species, *Haloarcula quadrata*, to accom-

modate this strain. For the time being, the species description is necessarily based on a single strain, as no additional square strains have been isolated and preserved to the best of our knowledge.

The finding that strain 801030/1^T is a *Haloarcula*, and so are the 'box-shaped' isolates originating from attempts to isolate square bacteria (Javor *et al*., 1982), does not imply that all square bacteria, including the gas vacuoles containing cells first described by Walsby (1980) and since found ubiquitously in hypersaline environments, are members of the *Haloarcula* genus. Most probably this is not the case, as shown by analyses of communities of square bacteria collected from their natural habitats. A sample of material from the Gavish Sabkha rich in square bacteria was analysed by fast atom bombardment-mass spectrometry. In addition to PG, PGP and PGS, a trisaccharide glycolipid and its sulfated derivative were found that were identical to the TGD-1 (1-*O*-[*β*-D-galactose-(1′ → 6′)-α-D-mannose-(1′ → 2′)-α-D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol) and S-TGD-1 (1-*O*-[*β*-D-galactose-(3′-SO_3H)-(1′ → 6′)-α-D-mannose-(1′ → 2′)-α-D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol) glycolipids of *Halobacterium salinarum* (Fredrickson *et al*., 1989). Polar lipid analysis of a brine sample from a saltern crystallizer pond in Eilat, Israel, in which the majority of the cells showed a square to trapezoid flat shape with gas vesicles, yielded PG, PGP, PGS and a single glycolipid, chromatographically identical with S-DGD-1 (1-*O*-[*β*-D-mannose-(6′-SO_3H)-(1′ → 2′)-α-D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol), the major glycolipid found in all *Haloferax* species (Oren *et al*., 1996). Therefore, it may tentatively be concluded that these square bacteria contained S-DGD-1 as their (principal) glycolipid. Thus, it may be assumed that the square morphology has originated in more than one genus of halophilic archaea. Whatever their taxonomic affiliation might be, the gas-vacuole-containing type of square bacteria observed by Walsby (1980), and described as non-motile (Grant & Larsen, 1989), has thus far defied all microbiologists' attempts to isolate them.

Description of *Haloarcula quadrata* (Oren, Ventosa, Gutiérrez and Kamekura) sp. nov.

Haloarcula quadrata (qua.dra′ta. L. fem. adj. *quadrata* square).

Gram-negative. Predominantly square-shaped, pleomorphic, flat cells, 2–3 μm, motile by means of one or more flagella. Colonies small, entire, smooth and red–orange. Chemo-organotrophic. Growth occurs in the presence of 2·7–4·3 M NaCl. At least 50–100 mM Mg^{2+} is required for optimal growth and maintenance of the square morphology. Optimum temperature for growth is 53 °C; no growth observed above 55 °C. Growth occurs on glucose, sucrose and maltose as single carbon and energy sources. Growth stimulation further observed by galactose, mannitol, sorbitol, glycerol, succinate and pyruvate. Acid is produced in the presence of glucose, galactose, sucrose, xylose or ribose. Grows anaerobically in the presence of nitrate with formation of nitrite and gas. No anaerobic growth in the presence of arginine. Starch hydrolysed. No hydrolysis of gelatin, casein, Tween 20 or Tween 80. Indole is not produced. Oxidase- and catalase-positive. Polar lipids are phosphatidylglycerol, the methyl ester of phosphatidylglycerophosphate, phosphatidylglycerosulfate and a single glycolipid (TGD-2). Susceptible to bacitracin, novobiocin and anisomycin (25 μg ml^{-1}). Resistant to penicillin, ampicillin, rifampicin, chloramphenicol, neomycin and erythromycin (50 μg ml^{-1}). The G+C content of the type strain is 60·1 mol% (T_m method). The type strain, 801030/1^T, was isolated from a brine pool in the Sinai peninsula, Egypt, and is deposited in the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as strain DSM 11927^T. The 16S rRNA sequence accession numbers of the type strain are AB010964 and AB010965.

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REFERENCES

Alam, M., Claviez, M., Oesterhelt, D. & Kessel, M. (1984). Flagella and motility behaviour of square bacteria. *EMBO J* **3**, 2899–2903.

Cohen, S., Oren, A. & Shilo, M. (1983). The divalent cation requirement of Dead Sea halobacteria. *Arch Microbiol* **136**, 184–190.

De Ley, J. & Tijtgat, R. (1970). Evaluation of membrane filter methods for DNA-DNA hybridization. *Antonie Leeuwenhoek* **36**, 461–474.

Ferragut, C. & Leclerc, H. (1976). Étude comparative des méthodes de détermination du T_m de l'ADN bactérien. *Ann Microbiol* (*Paris*) **127**, 223–235.

Fredrickson, H. L., Rijpstra, W. I. C., Tas, A. C., van der Greef, J., LaVos, G. F. & de Leeuw, J. W. (1989). Chemical characterization of benthic microbial assemblages. In *Microbial Mats: Physiological Ecology of Benthic Microbial Communities*, pp. 455–468. Edited by Y. Cohen & E. Rosenberg. Washington, DC: American Society for Microbiology.

González, C., Gutiérrez, C. & Ramirez, C. (1978). *Halobacterium vallismortis* sp. nov. An amylolytic and carbohydrate-metabolizing extremely halophilic bacterium. *Can J Microbiol* **24**, 710–715.

Grant, W. D. & Larsen, H. (1989). Extremely halophilic archaeobacteria. Order Halobacteriales ord. nov. In *Bergey's Manual of Systematic Bacteriology*, pp. 2216–2233. Edited by N. Pfennig. Baltimore: Williams & Wilkins.

Guixa-Boixareu, N., Calderón-Paz, J. I., Heldal, M., Bratbak, G. & Pedrós-Alió, C. (1996). Viral lysis and bacterivory as prokaryotic loss factors along a salinity gradient. *Aquat Microb Ecol* **11**, 215–227.

Gutiérrez, C. & González, C. (1972). Method for simultaneous detection of proteinase and esterase in extremely halophilic bacteria. *Appl Microbiol* **24**, 516–517.

Ihara, K., Watanabe, S. & Tamura, T. (1997). *Haloarcula argentinensis* sp. nov. and *Haloarcula mukohataei* sp. nov., two new extremely halophilic archaea collected in Argentina. *Int J Syst Bacteriol* **47**, 73–77.

Javor, B., Requadt, C. & Stoeckenius, W. (1982). Box-shaped halophilic bacteria. *J Bacteriol* **151**, 1532–1542.

Johnson, J. L. (1994). Similarity analysis of DNAs. In *Methods for General and Molecular Bacteriology*, pp. 655–681. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Juez, G., Rodriguez-Valera, F., Ventosa, A. & Kushner, D. J. (1986). *Haloarcula hispanica* spec. nov. and *Haloferax gibbonsii* spec. nov., two new species of extremely halophilic archaebacteria. *Syst Appl Microbiol* **8**, 75–79.

Kamekura, M. (1999). Diversity of members of the family Halobacteriaceae. In *Microbiology and Biogeochemistry of Hypersaline Environments*, pp. 13–25. Edited by A. Oren. Boca Raton: CRC Press.

Kamekura, M. & Seno, Y. (1993). Partial sequence of the gene for a serine protease from a halophilic archaeon *Haloferax mediterranei* R4, and nucleotide sequences of 16S rRNA encoding genes from several halophilic archaea. *Experientia* **49**, 503–513.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–219.

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.

Mylvaganan, S. & Dennis, P. P. (1992). Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. *Genetics* **130**, 399–410.

Oren, A. (1994). The ecology of the extremely halophilic archaea. *FEMS Microbiol Rev* **13**, 415–440.

Oren, A., Lau, P. P. & Fox, G. E. (1988). The taxonomic status of '*Halobacterium marismortui*' from the Dead Sea: a comparison with *Halobacterium vallismortis*. *Syst Appl Microbiol* **10**, 251–258.

Oren, A., Ginzburg, M., Ginzburg, B. Z., Hochstein, L. I. & Volcani, B. E. (1990). *Haloarcula marismortui* (Volcani) sp. nov., nom. rev., an extremely halophilic bacterium from the Dead Sea. *Int J Syst Bacteriol* **40**, 209–210.

Oren, A., Duker, S. & Ritter, S. (1996). The polar lipid composition of Walsby's square bacterium. *FEMS Microbiol Lett* **138**, 135–140.

Oren, A., Ventosa, A. & Grant, W. D. (1997). Proposal of minimal standards for the description of new taxa in the order *Halobacteriales*. *Int J Syst Bacteriol* **47**, 233–238.

Owen, R. J. & Hill, L. R. (1979). The estimation of base compositions, base pairing and genome size of bacterial deoxyribonucleic acids. In *Identification Methods for Microbiologists*, 2nd edn, pp. 217–296. Edited by F. A. Skinner & D. W. Lovelock. London: Academic Press.

Owen, R. J. & Pitcher, D. (1985). Current methods for estimating DNA base composition and levels of DNA-DNA hybridization. In *Chemical Methods in Bacterial Systematics*, pp. 67–93. Edited by M. Goodfellow & E. Minnikin. London: Academic Press.

Parkes, K. & Walsby, A. E. (1981). Ultrastructure of a gas-vacuolate square bacterium. *J Gen Microbiol* **126**, 503–506.

Peijin, Z., Yi, X., Changsong, X., Yunquing, M. & Hongdi, L. (1994). New species of *Haloarcula*. *Acta Microbiol Sin* **34**, 89–95.

Stoeckenius, W. (1981). Walsby's square bacterium: fine structure of an orthogonal procaryote. *J Bacteriol* **148**, 352–360.

Takashina, T., Hamamoto, T., Otozai, K., Grant, W. D. & Horikoshi, K. (1990). *Haloarcula japonica* sp. nov., a new triangular halophilic archaebacterium. *Syst Appl Microbiol* **13**, 177–181.

Tindall, B. J. (1992). The family *Halobacteriaceae*. In *The Prokaryotes*, 2nd edn, pp. 768–808. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Torreblanca, M., Rodriguez-Valera, F., Juez, G., Ventosa, A., Kamekura, M. & Kates, M. (1986). Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipid composition, and description of *Haloarcula* gen. nov. and *Haloferax* gen. nov. *Syst Appl Microbiol* **8**, 89–99.

Torrella, F. (1986). Isolation and adaptive strategies of haloarculae to extreme hypersaline habitats. In *Abstracts of the Fourth International Symposium on Microbial Ecology*, p. 59. Ljubljana: Slovene Society for Microbiology.

Walsby, A. E. (1980). A square bacterium. *Nature* **283**, 69–71.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Xu, Y., Liu, H. & Zhou, P. (1995). Nucleotide sequence of the 16S rRNA from an archaea, *Haloarcula aidinensis* strain B2. *Acta Microbiol Sin* **35**, 77–85.